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Abbreviation List

ANOVA, analysis of variance

As-MECs, arsenic-transformed malignant epithelial cells

Cd-MECs, cadmium-transformed malignant epithelial cells

cDNA: complementary DNA

CSCs, cancer stem cells

DAPI, 4', 6'-diamideino-2-2phenylindole

ELISA, enzyme-linked immunosorbent assay

EMT, epithelial-to-mesenchymal transition

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

KSFM, keratinocyte serum-free medium

LSD, Least significant differences

MECs, malignant epithelial cells

MMP, matrix metalloproteinase

MNU-MECs, N-methyl-N-nitrosourea-transformed malignant epithelial cells

NSCs, normal stem cells

RT-PCR, real-time reverse transcription-polymerase chain reaction

SCs, stem cells

SE, standard error

Abstract

BACKGROUND: Cancer stem cells (CSCs) drive tumor initiation, progression and metastasis. Microenvironment is critical to the fate of CSCs. We have found that a normal stem cell (NSC) line from human prostate (WPE-stem) is recruited into CSC-like cells by nearby, but noncontiguous arsenic-transformed isogenic malignant epithelial cells (MECs).

OBJECTIVE: It is unknown if this recruitment of NSCs into CSCs by non-contact co-culture is specific to arsenic-transformed MECs. Thus, here, we tested the effects of neighboring noncontiguous cadmium-transformed MECs (Cd-MECs) and N-methyl-N-nitrosourea-transformed MECs (MNU-MECs) co-culture on NSCs.

RESULTS: After two weeks of non-contact Cd-MEC co-culture, NSCs showed elevated metalloproteinase-9 (MMP-9) and MMP-2 secretion, increased invasiveness, increased colony formation, decreased *PTEN* expression and formation of aggressive, highly branched ductal-like structures from single cells in Matrigel, all characteristics typical of cancer cells. These oncogenic characteristics did not occur if NSCs were co-cultured with MNU-MECs. The NSCs co-cultured with Cd-MECs retained self-renewal capacity as evidenced by multiple passages (>3) of structures formed in Matrigel. Cd-MEC co-cultured NSCs also showed molecular (increased *VIMENTIN*, *SNAIL1* and *TWIST1* expression; decreased *E-CADHERIN* expression) and morphologic evidence of epithelial-to-mesenchymal transition typical for conversion to CSCs. Dysregulated expression of SC-renewal genes, including *ABCG2*, *OCT-4* and *WNT-3*, also occurred in NSCs during oncogenic transformation induced by non-contact co-culture with Cd-MECs.

CONCLUSIONS: These data indicate that Cd-MECs can recruit nearby NSCs into a CSC-like phenotype, but MNU-MECs do not. Thus, the recruitment of NSCs into CSCs by nearby MECs is dependent on the carcinogen originally used to malignantly transform the MECs.

Introduction

In recent years, the hypothesis of the existence of cancer stem cells (CSCs) has helped provide an explanation for tumor initiation, progression, therapeutic resistance and tumor recurrence (Visvader and Lindeman 2008). CSCs have been found in or isolated from a variety of tumors (Visvader and Lindeman 2008). Typically a small subpopulation of the total tumor cells, like normal stem cells (NSCs) CSCs have the capacity for self-renewal, and an unlimited capacity for differentiation. CSCs share other characteristics with NSCs (Pardal et al. 2003), and, like NSCs, CSCs reside in a niche (Borovski et al. 2011). Neighboring cells influence the differentiation and homeostasis of nearby stem cells (SCs) by releasing soluble factors, such as growth factors, chemokines, and cytokines into the microenvironment (Abbott et al. 2008). However, compared with NSCs, CSCs have dysregulated self-renewal programming and are genetically unstable (Adams and Strasser 2008; Bomken et al. 2010; Pardal et al. 2003). The origin of CSCs is debated but they may originate from adult SCs, partially differentiated progenitor cells or even differentiated cells with multiple genetic and/or epigenetic alterations (Wang 2010). In addition, CSCs and more differentiated cancer cells may exist in a dynamic equilibrium, subject to a bidirectional conversion based on microenvironmental factors (Li and Laterra 2012).

SCs may play an important role in cancer induced by agents in the human environment. We find a survival selection of SCs occurs causing an overabundance of CSCs as exposure to the human carcinogen, arsenic, induces cancer *in vivo* or causes acquisition of malignant phenotype *in vitro* (Sun et al. 2012; Tokar et al. 2010a; Tokar et al. 2010b; Waalkes et al. 2008). In addition, arsenic directly transforms the NSCs into CSCs *in vitro* (Tokar et al. 2010a; Tokar et al. 2013). Arsenic can also indirectly induce a CSC-like phenotype when NSCs are co-cultured with nearby, but noncontiguous, arsenic-transformed malignant epithelial cells (As-MECs), even

though no actual physical cell-to-cell contact occurs (Xu et al. 2012). The non-contact conversion of NSCs to CSCs appears to involve soluble factors secreted by neighboring As-MECs and does not involve remnant arsenic (Xu et al. 2012). This recruitment of NSCs into CSCs by non-contact co-culture with MECs could have important general implications in tumor growth, invasion and dissemination, but has only been observed with MECs originally transformed by arsenic (Xu et al. 2012).

Cadmium is a widespread environmental contaminant and an important human carcinogen (IARC 2012). Cadmium exposure is associated with increased risk of cancer, as well as cardiovascular, kidney and bone disease (IARC 2012; ATSDR 2008). In addition to occupational exposure, human exposure to cadmium mainly occurs through food consumption (in particular cereals and vegetables), tobacco use and inhalation of ambient air (ATSDR 2008).

The mechanism of cadmium carcinogenesis likely differs from another important human inorganic carcinogen, arsenic (Achanzar et al. 2002; Tokar et al. 2010b). When used in transforming the same non-tumorigenic human prostate epithelial cell line, RWPE-1, cadmium requires much less time than inorganic arsenic (8 versus 29 weeks) (Achanzar et al. 2002; Achanzar et al. 2001). However, the production of CSC-like spheres, holoclones and colony forming capacity induced by cadmium-transformation of RWPE-1 cells, which are all indicative of CSC formation, is much lower than that produced by arsenic-transformed RWPE-1 cells (Tokar et al. 2010b). Other evidence indicates cadmium treatment leads to an over-abundance of SCs and blocks differentiation during the malignant transformation of human breast epitheial cells (Benbrahim-Tallaa et al. 2009). Chronic cadmium exposure inducing cancer cell characteristics in human pancreatic cells increases CSC-like cells (Qu et al. 2012). However, in

one study, cadmium inhibited mouse prostate stem/progenitor cell proliferation and self-renewal (Jiang et al. 2011).

The recruitment of NSCs into CSCs by As-MECs could be an important general characteristic of MECs, but has not been shown for malignant epithelia induced by carcinogens other than inorganic arsenic (Xu et al. 2012). Thus, in this study we determined if cadmium-transformed MECs (Cd-MECs) had the ability to impact neighboring but noncontiguous NSCs. In addition, NSCs co-cultured with N-methyl-N-nitrosourea (MNU)-transformed MECs (MNU-MECs) were also tested to compare organic and inorganic carcinogens.

Materials and Methods

Cell lines and culture

RWPE-1 cells are an immortalized non-tumorigenic human prostate epithelia line derived from the non-neoplastic adult prostate (Bello et al. 1997). The normal prostate stem cell line, WPE-stem cells (termed NSCs hereafter), isolated from RWPE-1 cells by single-cell dilution cloning, is well established as a NSC line showing multiple typical characteristics of urogenital system stem/progenitor cells (Tokar et al. 2005; Tokar et al. 2010a). Cadmium malignantly transformed prostate epithelia (originally termed CTPE cells; in this study termed Cd-MECs) were developed from RWPE-1 cells chronically exposed to cadmium (10 μM, 8 weeks). These Cd-MECs show loss of contact inhibition and elevated secretion of MMP-9 and MMP-2 *in vitro* and form highly invasive tumors in mice (Achanzar et al. 2001). WPE1-NB26 cells (termed as MNU-MECs in this study) are derived from second generation tumors formed in nude mice by MNU-transformed RWPE-1 cells (10 μg/ml MNU for 1 h, four cycles). The MNU-MECs are

tumorigenic *in vivo* (Webber et al. 2001). Thus, all the cell lines used in the present study are isogenic.

Cells were maintained in the low-calcium serum free medium [keratinocyte serum-free medium (KSFM)] containing 50 μg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, and 1% antibiotic-antimycotic mixture (Gibco, Rockville, MD). The effects of neighboring Cd- or MNU-MECs on the NSCs were tested via co-culture transwell, using inserts, which does not allow cell-to-cell contact but allows soluble factors to pass and places the two types of cells apart by the width of approximately 50-100 normal prostate epithelia. Cell culture and co-culture were conducted as described previously (Xu et al. 2012).

Free-floating sphere formation

Floating sphere formation in culture is common for NSCs or CSCs (Ponti et al. 2005). After MEC co-culture, 1000 SCs were plated in each well of an uncoated 6-well plate and fed every 48 hrs. After 1 week, floating spheres and adherent cells were collected separately and stained with Trypan Blue (Sigma-Aldrich, St. Louis, MO). Viable free-floating spheroids and adherent cells from each well were quantitated by microscopic visual counting or automated cell counter, respectively, and processed further as needed.

Matrix metalloproteinase activity

After MEC co-culture, SCs were grown alone to collect 48-hr conditioned medium and cells were counted. Secreted MMP-9 and MMP-2 activity was examined in medium, by zymography (Tokar et al. 2005) and then adjusted to cell number. In some cases, conditioned medium from viable free-floating spheroid cells after MEC co-culture was assessed for MMP activity.

Branched ductal-like structures and serial passage

After 2 weeks of co-culture, floating sphere cells were dissociated with 0.05% trypsin-EDTA, filtered with 40-µm strainer to get the single cell suspension, re-suspended in Matrigel (BD Biosciences, Bedford, MA) with KSFM (1:1 V:V), plated in the 24-well plate and incubated at 37 °C overnight to solidify before 1 ml KSFM was added. Medium was changed every 3 days. Images were taken via inverted microscope after 2 weeks. For serial passage, a single colony in Matrigel was dissociated into single cells and replated in Matrigel-KSFM mixture as above.

Anchorage-independent growth

Colony formation in soft agar was performed as described (Tokar et al. 2005) on cells from free-floating spheres.

Invasion assay

Invasive capacity was assessed using a modified Boyden chamber assay as described (Bello et al. 1997).

Real-time reverse transcription–polymerase chain reaction

Total RNA, isolated from cultures with TRIzol (Invitrogen, Carlsbad, CA) and purified with RNeasy mini kit columns (Qiagen, Valencia, CA), was reverse transcribed to complementary DNA (cDNA) using Moloney murine leukemia virus reverse transcriptase (Applied Biosystem, Foster, CA). The resulting cDNAs were subjected to real-time reverse transcription-polymerase chain reaction (RT-PCR) for *PTEN*, *VIMENTIN* (*VIM*), *E-CADHERIN* (*E-CAD*), *SNAIL1*, *TWIST1*, *ABCG-2*, *OCT-4* and *WNT-3* as described (Tokar et al. 2010a). Sequences of gene-specific primers (Sigma-Aldrich, St. Woodlands, TX) are provided in Supplemental Material, Table S1.

Western blots

Western blots were conducted as described (Tokar et al. 2010b) using an antibody against PTEN (Abcam, Cambridge, MA). Protein bands were detected with the SuperSignal Chemiluminescence Substrate (Thermo Scientific, Rockford, IL).

Immunofluorescence

Immunofluorescence was conducted in Lab-Tek Chamber Slide (Electron Microscopy Sciences, Hatfield, PA) as described (Xu et al. 2012). After MEC co-culture, SCs were plated in the chamber and fixed with acetone and methanol (1:1, V:V), blocked with normal horse serum (1:60 in PBS, room temperature, 1 hr), incubated with primary antibody [mouse anti-VIMENTIN (Sigma-Aldrich, St. Louis, MO)] at 4°C, overnight, and then incubated with secondary antibodies labeled with Alexa Fluor 568 (Molecular Probes, Eugene, OR), at room temperature for 1 hr. After washing with PBS, cells were stained with 4', 6'-diamideino-2-2phenylindole (DAPI) (Invitrogen, Eugene, OR) and examined via inverted fluorescence microscope.

TGF-β1 analysis

After MEC co-culture for 2 weeks, medium was analyzed by enzyme-linked immunosorbent assay (ELISA) array with human autoimmune response multi-analyte ELISA Array kit (Qiagen, Valencia, CA) and Human TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN). NSCs were treated with 10 ng/ml of TGF-β1 (Peprotech, Rocky Hill, NJ) for 96 hrs and tested for MMP secretion, cell morphology, and target gene expression.

Statistical analysis

Data represent the mean and standard error (SE) of 3 or more separate comparisons. For single comparison to control, Student's *t*-test was used. The least significant differences (LSD) test after analysis of variance (ANOVA) was used for multiple comparisons. A $p < 0.05$ was considered to be significant.

Results

Oncogenic transformation of NSCs

We found that non-contact co-culture with As-MECs could recruit NSCs into CSCs in our prior work. To see if this was arsenic-specific, we tested Cd-MECs and MNU-MECs in co-culture with NSCs. During chemically-induced malignant transformation, cells identical to or isogenic with the NSCs used in this study produce xenograph tumors in mice when secreted MMP-9 activity is increased 200% to 450% of control (Achanzar et al. 2002; Tokar et al. 2010a; Tokar et al. 2010b). After non-contact co-culture with Cd-MECs for 2 weeks, SCs showed increased secretion of both MMP-9 (360% of control) and MMP-2 (220% of control) (Figure 1A). Similar results were observed when the same NSCs were co-cultured with As-MECs in prior work. NSCs co-cultured with MNU-MECs showed no change in MMP secretion (Figure 1A).

Free-floating sphere formation is common for NSCs and CSCs. We often see increased sphere formation with arsenic-induced CSC formation. However, after Cd-MEC or MNU-MEC non-contact co-culture, floating sphere formation was unchanged (Figure 1B). Floating spheres, often enriched in NSCs or CSCs, from Cd-MEC co-culture did show elevated MMP-9 (150% of control) and MMP-2 (420% of control) secretion (Figure 1C) indicating an aggressive phenotype.

Cells derived from spheres generated from SCs co-cultured with Cd-MECs for 2 weeks showed a marked increase in invasion, indicating aggressive phenotype, while MNU-MEC co-culture did not show enhanced invasion (Figure 2A).

Anchorage-independent growth (colony formation) is also common for SCs and an indicator of aggressive phenotype. After Cd-MEC co-culture, the spheroid derived SCs formed more colonies than control, but not after MNU-MEC co-culture (Figure 2B).

After Cd-MEC co-culture, sphere derived SCs produced highly branched ductal-like, aggressive structures from single cells in Matrigel (Figure 2C). The structures formed after Cd-MEC co-culture could be serial passaged for at least 4 times, confirming self-renewal capacity. Spheroids derived MNU-MEC co-culture or control SCs also formed structures in Matrigel, which could be serial passaged, but the structures were small and showed minimal branching (Figure 2C). Thus, the SCs retained their self-renewal capacity after co-culture regardless of the carcinogen originally transforming the MECs, but gained an aggressive phenotype only with Cd-MEC co-culture.

PTEN suppression and epithelial-to-mesenchymal transition

PTEN is a tumor suppressor and has a key role in SC self-renewal and differentiation. A rapid suppression of *PTEN* expression occurred in SCs with Cd-MEC co-culture (Figure 3A, transcript levels, Figure 3B, protein levels), but not with MNU-MEC co-culture. Thus, at this point we felt it was clear that Cd-MEC co-culture lead to acquired cancer characteristics and further in-depth study on Cd-MEC co-cultured SCs was justified.

Epithelial-to-mesenchymal transition (EMT) is widely observed in cancer and plays a role in invasion and metastasis. After Cd-MEC co-culture SCs took on a morphology (spindle-shaped,

etc.) typical of mesenchymal cells, indicating EMT had occurred (Figure 4A). With Cd-MEC co-culture, SCs showed a marked and wide-spread increase in VIM protein, a mesenchymal cell marker (Figure 4B), and increased *VIM* transcript (Figure 4C). Transcript of *SNAIL1* (Figure 4D) and *TWIST1,* (Figure 4E), EMT-inducing transcription factors, increased, while *E-CAD* transcript, an epithelial marker, decreased (Figure 4F).

SC-related gene expression

A "U-shaped" expression of the prostate SC-associated genes occurs in SCs during malignant transformation by either direct arsenic exposure or As-MEC co-culture (Tokar et al. 2010a; Xu et al. 2012). Cd-MEC co-cultured SCs also showed a "U-shaped" expression in the SC-related genes, *ABCG-2*, *OCT-4* and *WNT3*. The levels of these genes initially decreased with Cd-MEC co-culture at 1 week and rebounded above control after 2 weeks (Figure 5).

Role of TGF-β1 in MEC co-culture

Potential soluble factors that could be secreted by MECs and impact transformation by MEC co-culture were assessed by ELISA. High TGF-β1 levels occurred in As- and Cd-MEC co-culture medium, but not in MNU-MEC medium (Figure 6A). Directly treating NSCs with TGF-β1 induced similar responses as with Cd-MEC co-culture, such as increased MMP secretion (Figure 6B), EMT (Figure 6C) and gene expression changes like decreased *E-CAD* and increased *VIM* transcript (Figure 6D).

Discussion

CSCs are hypothesized to be responsible for tumor initiation, progression, and metastasis (Visvader and Lindeman 2008). CSCs share many properties with NSCs, such as self-renewal

capacity and differentiation potential, but in a distorted fashion. Clearly, the CSC microenvironment, which is composed of others cells, the extracellular matrix and secreted factors, is key to their behavior (Moore and Lemischka 2006). Recently, we found NSCs could be driven into a CSC-like phenotype by nearby, yet noncontiguous isogenic As-MECs (Xu et al. 2012). This study used a non-contact, co-culture technique which let only soluble, secreted factors from As-MECs interact from the NSCs over an approximate distance of 50 to 100 normal prostate cells (Xu et al. 2012). Essentially, this constitutes a recruitment of critical cells involved in tumor formation or replenishment by distant malignant epithelia, which was, in fact, made originally malignant by the important human carcinogen, inorganic arsenic (Xu et al. 2012). In the present study, CSC recruitment also occurred with epithelial cells transformed by cadmium, another wide-spread human environmental carcinogen. So for both arsenic and cadmium, this CSC recruitment could be an important mode of tumor expansion. Further, we tested Cd- and MNU-MECs, and found only Cd-MEC co-culture could recruit NSCs into CSCs. The recruited cells kept basic SC characteristics, like self-renewal ability and sphere forming capacity, during the acquisition of oncogenic phenotype. In contrast, MNU-MECs did not recruit NSC into an oncogenic phenotype. This is further evidence that human prostate NSCs can be recruited into CSC-like cells via microenvironment containing substances secreted by MECs, but only by MECs originally transformed by some (inorganic arsenic and cadmium) but not all (MNU) carcinogens. This recruitment phenomenon may be a very important mechanism in chemically-induced tumor extension and dissemination, with cancer cells induced by some chemical carcinogens.

In the tumor microenvironment, surrounding tissues and tumors exchange information either directly through cell-to-cell contact or indirectly through chemical signaling from nearby cells

(Bissell and Radisky 2001). In the co-culture system we used, there was no physical contact between SCs and MECs, making direct cell-to-cell information relay impossible. Though Cd-MECs were transformed by cadmium, which could accumulate and then possibly efflux, the cells used in this study were passed multiple times prior to the initial experiment and when tested, the amount of cadmium effluxed by Cd-MECs into the co-culture medium did not exceed background as detected by atomic absorption spectrometry (not shown). Thus, the recruitment by Cd-MEC co-culture of CSC-like cells seems likely due to the secreted factors that pass from one chamber to the other. As-MECs (Xu et al. 2012) and Cd-MECs (present study) seem capable of inducing nearby NSCs into a cancer-like phenotype, while MNU-MECs are not. The reasons for these differences are not immediately apparent. MNU is a strong genotoxicant acting by direct alkylation of DNA resulting in mutations (Robbiano et al. 1989), and induces malignant transformation *in vitro* in only a few hours (Webber et al. 2001). The mechanisms of arsenic and cadmium carcinogenesis are incompletely defined, though both likely involve multiple factors including genetic and epigenetic changes, oxidative stress and inflammation (IARC 2012). Chronic arsenic exposure induces stress and inflammation which may contribute to cancer (Mo et al. 2011). Inflammation caused by chronic arsenic exposure can persist over time in spite of changes in exposure levels (Chen et al. 2007). Cadmium-induced tissue damage is often accompanied by inflammation involving cytokine production (Kundu et al. 2009). As- and Cd-MECs were produced from chronic, low-level exposure (Achanzar et al. 2002; Achanzar et al. 2001), and likely involve chronic reactions possibly including production of inflammatory factors that could be secreted into the media, which in turn affect a SC microenvironment. Indeed, we saw that As-MECs secreted IL-6, which mimicked the CSC recruiting effects of As-MECs co-culture (Xu et al. 2012). Inflammatory factors are important constituents of the tumor

microenvironment, and inflammation is recognized a key factor in cancer (Kim et al. 2009; Mantovani et al. 2008). Cd-MECs did not show elevated secretion of IL-6 (data not shown). However, TGF-β1was highly secreted by both As-MECs and Cd-MECs, but not by MNU-MECs which did not recruit NSCs into CSCs. Studies demonstrate that TGF-β positively regulates tumor progression and metastasis (Bierie and Moses 2006). TGF-β can also induce EMT (de Graauw et al. 2010). Direct TGF-β1 treatment of NSCs duplicated in several ways CSC recruitment by Cd-MECs, stimulating MMP secretion and EMT, suggesting it may be another key signaling factor in the recruitment of NSCs into CSCs induced by some MECs depending on the transforming carcinogen. The involvement of signaling factors in this CSC recruitment deserves further study, although there are very likely other important facets to this complex phenomenon.

EMT converts epithelial cells into cells with mesenchymal traits, involving loss of cell-cell adhesion, altered polarity, and increased motility (Kalluri and Weinberg 2009). EMT is important for tumor initiation, progression and metastasis. Indeed, various epithelia cell lines, including the prostate epithelial cells, undergo EMT during malignant transformation (Coppola et al. 2012). The EMT inducer TWIST1 promotes tumor development in mice and fosters malignant transformation of human mammary epithelial cells (Morel et al. 2012). Multiple studies show that EMT promotes the generation of CSCs (Mani et al. 2008; Morel et al. 2008). The induction of EMT by *SNAIL* and *TWIST* transfection or TGFβ treatment in mammary epithelial cells results in cells with multiple CSC characteristics (Mani et al. 2008), consistent with our observation of *SNAIL* and *TWIST* over-expression during Cd-MEC co-culture recruitment of CSC-like cells. We also saw increased mesenchymal marker, *VIM*, and loss of epithelial maker, *E-CAD*, indicating EMT. Thus, we found NSCs acquire the morphology and

molecular signs of EMT as they acquired a CSC-like phenotype from Cd-MEC co-culture. These data support the concept that EMT occurs during MEC recruitment of NSCs into CSCs.

During Cd-MEC co-culture, SCs showed decreased expression of the tumor suppressor gene *PTEN*. In prostate cancers, the loss of *PTEN* expression often occurs (Verhagen et al. 2006), and is related to the selection and expansion of SCs during malignant transformation (Tokar et al. 2010b). Similar to what occurred in As-MEC co-culture (Xu et al. 2012), some SC self-renewal genes, *ABCG-2*, *OCT-4* and *WNT*-3, showed dysregulated expression during the acquisition of CSC-like characteristics after Cd-MEC co-culture. *ABCG-2* is thought to be a survival factor of SCs, ultimately driving tumor growth (Dean et al. 2005). Both *OCT-4* and *WNT* regulate SC self-renewal, potentially drive the acquisition of CSC-like properties and are linked to carcinogenesis (Hochedlinger et al. 2005; Vermeulen et al. 2010). This early loss and subsequent reactivation of critical SC-related gene expression occurred in arsenic-induced malignant transformation of WPE-stem cells (Tokar et al. 2010a) and transformation of hematopoietic SCs into leukemic SCs (Krivtsov et al. 2006). Dysregulated SC self-renewal programming is typical for oncogenesis (Pardal et al. 2003). Thus, the distorted expression of SC-related genes is likely an important feature of CSC formation from NSCs.

Conclusions

Taken together, our data provide evidence that NSCs can acquire CSC-like characteristics via a microenvironment containing soluble factors derived from MECs originally transformed by the human environmental carcinogen, cadmium. Inflammatory factors like TGF-β1 may play a key role in this recruitment. The recruitment of NSCs into CSCs by nearby MECs does not apply to all carcinogens originally inducing the MECs, and the reasons for this difference should be

further explored. This CSC recruitment may be a very important aspect of carcinogenesis which facilitates chemically-induced tumor growth, invasion, and dissemination for some human carcinogens.

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Figure legends

Figure 1. Secreted MMP activity and non-adherent sphere formation after 2 weeks of co-culture with Cd-MECs or MNU-MECs. (A) Secreted MMP activity in SCs. (B) Sphere formation. (C) Secreted MMP activity by spheroid cells. Quantitative data presented as mean ± SE, n = 3. * $p < 0.05$, compared with control.

Figure 2. The cancer characteristics acquired by SCs after 2 weeks of co-culture with Cd-MECs or MNU-MECs. (A) Invasion capacity of spheroid SCs. (B) Colony formation of spheroid SCs. (C) Ductal-like structures formed in Matrigel over 2 weeks by spheroid single SCs after co-culture (bar = 200 μm). Quantitative data presented as mean ± SE, n = 6. * $p < 0.05$, compared with control.

Figure 3. Tumor suppressor gene *PTEN* expression. (A) Transcript levels of *PTEN* after 1 week and 2 weeks of co-culture. (B) Protein after 2 weeks of co-culture. Data presented as mean ± SE, n = 3. * $p < 0.05$, compared with control.

Figure 4. EMT of SCs induced by 2 weeks of Cd-MEC co-culture. (A) SC morphology (bar = 50 μm). (B) Protein levels of mesenchymal cell marker VIM (bar = 20 μm). (C) *VIM* transcript. (D) Transcript of EMT inducer *SNAIL1*. (E) Transcrip of EMT inducer *TWIST1*. (F) Transcript of epithelial cell marker *E-CAD*. Quantitative data presented as mean ± SE, n = 3. * $p < 0.05$, compared with control.

Figure 5. Expression of SC-related genes after 2 weeks of Cd-MEC co-culture. The deactivation and reactivation of (A) *ABCG-2*, (B) *OCT-4*, (C) *WNT*-3. Quantitative data presented as mean ± SE, n = 3. * $p < 0.05$, compared with control.

Figure 6. Possible involvement of TGF-β1 in recruitment of NSCs into CSCs by Cd- or As-MEC co-culture. (A) Secretion levels of TGF-β1 in co-culture medium at week 2. Direct treatment with 10 ng/ml of TGF-β1 on NSCs for 96 hrs: (B) enhanced secreted MMP activity in TGF-β1 treated SCs; (C) changed SC morphology (bar = 100 μm); and (D) altered transcript levels of *VIM* and *E-CAD*. Quantitative data presented as mean ± SE, n = 3. * $p < 0.05$, compared with control.

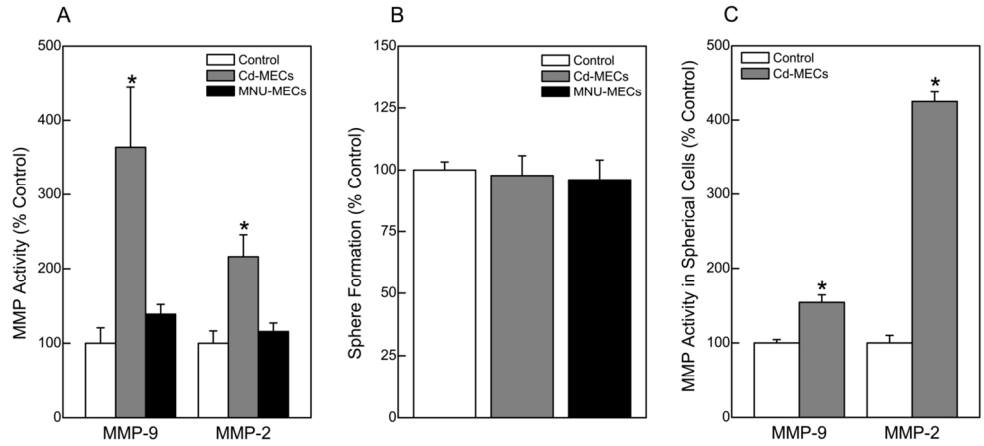

Figure 1

Figure 1

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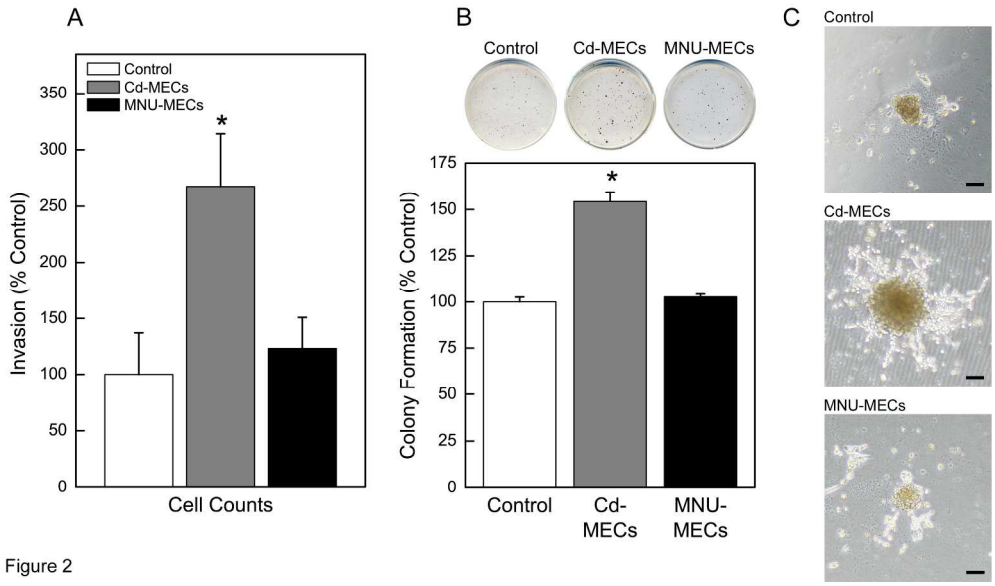

Figure 2

Figure 2
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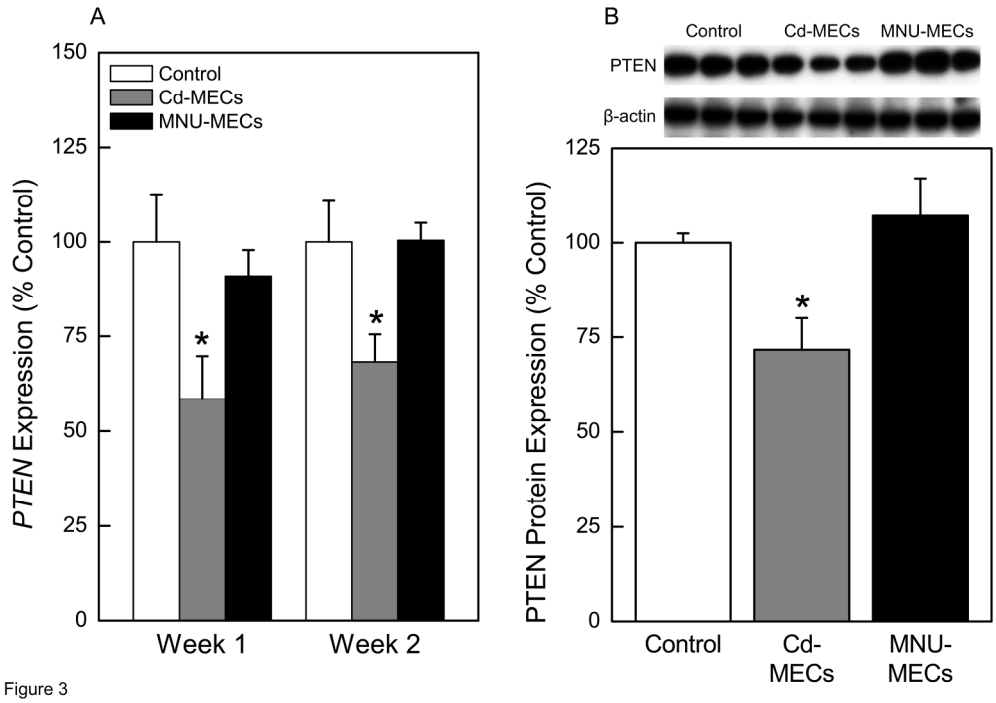

Figure 3

Figure 3

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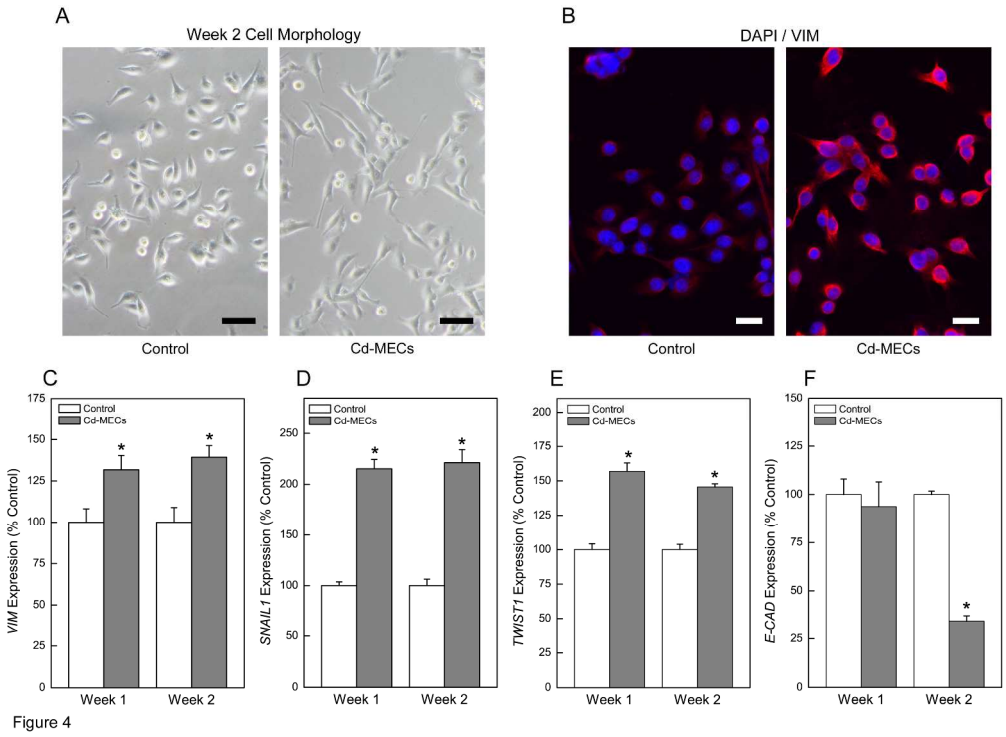

Figure 4

Figure 4

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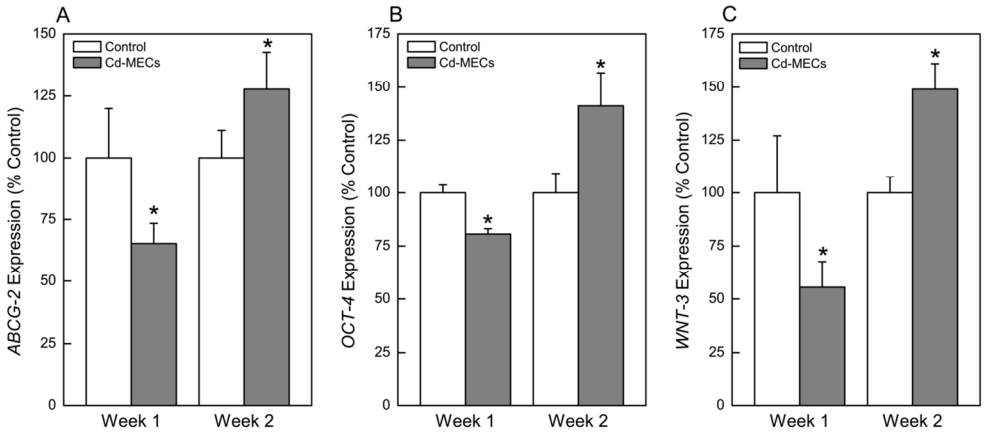

Figure 5

Figure 5

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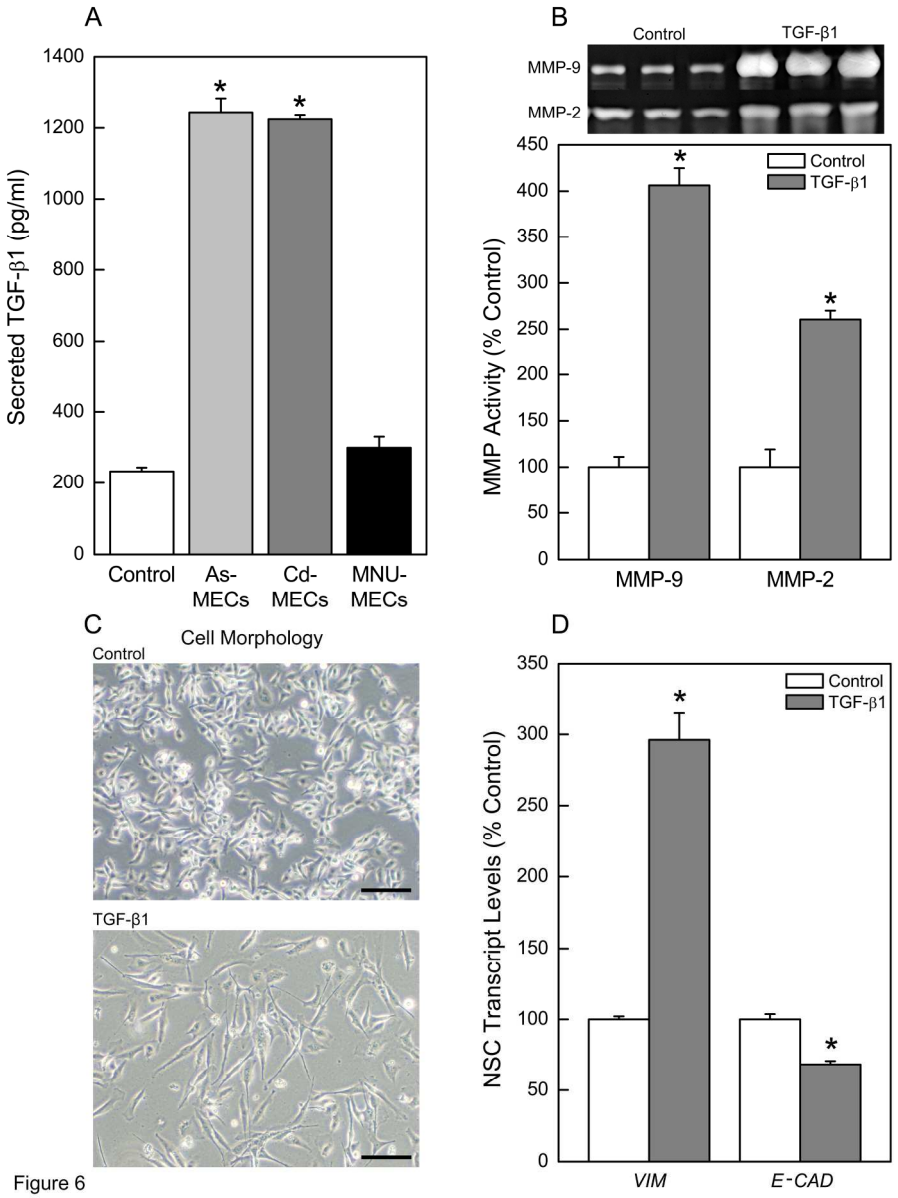

Figure 6

Figure 6
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